

Antibacterial Activities of Phenolic Benzaldehydes and Benzoic Acids against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*

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ABSTRACT

We evaluated the bactericidal activities of 35 benzaldehydes, 34 benzoic acids, and 1 benzoic acid methyl ester against *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* when these compounds were substituted on the benzene ring with 0, 1, 2, or 3 hydroxy (OH) and/or methoxy (OCH₃) groups in a pH 7.0 buffer. Dose-response plots were used to determine the percentage of the sample that induced a 50% decrease in CFU after 60 min (BA₅₀). Of the 70 compounds tested, 24 were found to be active against all four pathogens, and additional 4, 10, and 12 were found to be active against three, two, and one of the pathogens, respectively. *C. jejuni* was ~100 times as sensitive as the other three pathogens. The 10 compounds that were most active against the four pathogens (with average BA₅₀ values ranging from 0.026 to 0.166) and are candidates for studies of activity in foods or for disinfections were 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 2,3,4-trihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, 2,3-dihydroxybenzaldehyde, 2-hydroxy-3-methoxybenzaldehyde, 4-hydroxy-2,6-dimethoxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 2,4-dihydroxybenzaldehyde, and 2-hydroxybenzaldehyde. Comparison of the chemical structures of the test compounds and their activities revealed that (i) the aldehyde (CHO) group was more active than the carboxyl (COOH) group whether or not OH groups were present; (ii) compounds were most active with trisubstituted OH > disubstituted OH > monosubstituted OH; (iii) for disubstituted derivatives, 2-OH enhanced activities were exhibited by benzaldehyde but not by benzoic acid; (iv) compounds were more active with OH than with OCH₃, irrespective of the position of substitution on the benzene ring; (v) compounds with mixed OH and OCH₃ groups exhibited variable results, i.e., in some cases OCH₃ groups enhanced activity and in other cases they did not; (vi) methoxybenzoic acids were largely inactive; and (vii) gallic acid was 20 times as active against *S. enterica* at pH 7.0 as it was at pH 3.7, suggesting that the ionization of its OH groups may enhance bactericidal activity.

Pathogenic strains of *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* are linked to foodborne illnesses. Nontoxic food-compatible natural products are a source of compounds that may be useful in interventions to reduce pathogens on surfaces of fruits and vegetables and in antimicrobial formulations added to foods such as fruit and vegetable juices, meat, and poultry for disinfection or to minimize the contamination of these foods during storage and during commercial and home processing.

Phenolic compounds are secondary plant metabolites found in numerous plant species, and they play key roles in the biochemistry and physiology of plants. These compounds, especially oxidation products of phenolic compounds, appear to be involved in the defense of plants against invading pathogens, including bacteria, fungi, and viruses. In addition, polyphenol-oxidase-catalyzed polymerization of monomeric phenols such as chlorogenic acid helps to seal the injured plant surface and initiates the healing process, analogous to the formation of fibrin blood clots in injured animals and humans (12).

Dietary phenolic compounds have also been shown to

have numerous beneficial properties by virtue of their ability to act as antioxidants, anticarcinogens, antimutagens, and antiglycemic agents (19, 27, 37, 40–42). These properties can be used in the prevention of rancidity in food and in the development of health-promoting food ingredients. However, phenolic compounds can also darken protein-containing food ingredients and reduce the nutritional quality of protein through their ability to participate in polyphenol-protein enzymatic browning reactions catalyzed by polyphenol oxidases (11, 13).

In addition to their cited beneficial effects, phenolic compounds are also reported to have antimicrobial activities against human pathogens. The literature contains numerous studies on the antibacterial, antifungal, and antiviral activities of natural and synthetic phenolic compounds (1–5, 7–9, 16, 18, 20, 23–25, 27, 29, 31–35, 39, 44, 45), and these studies go back to the 19th century following the discovery by Lister and others that carbolic acid (phenol) can act as an antibiotic against wound infections (21). As described in detail in the excellent reviews by Davidson (6) and Fung et al. (17), the synthetic methyl and propyl esters of *p*-hydroxybenzoic acid, the so-called parabens, as well as tertiary butylhydroxyanisole (BHA) and tertiary butylhydroquinone (TBC), are on the generally-recognized-as-safe list for use in food at a maximum concentration of

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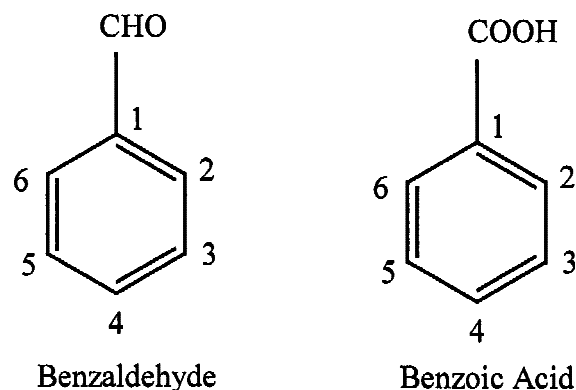


FIGURE 1. Structures of benzaldehydes and benzoic acids evaluated for antibacterial activity. Table 1 shows the positions of the OH and OCH₃ groups on the benzene rings.

0.02% (38). However, they appear not to be widely used, possibly because of their low solubility in water.

To assess the antimicrobial potential of natural compounds and plant extracts, we previously evaluated the relative bactericidal activities of 96 plant essential oils and 23 of their active components by testing them separately against the four major human pathogens (14). In addition, we have been evaluating other compounds in a broad effort to define the chemical basis for the antimicrobial activities of different classes of natural products. As part of this effort, the general objective of the present study was to screen a variety of benzaldehydes and benzoic acids substituted on the benzene ring with one, two, and three OH and/or OCH₃ groups (Fig. 1). The data obtained in this study indicate that the benzaldehydes are more active than the benzoic acids and that bactericidal activities are influenced by both the number and the position of the OH and OCH₃ groups on the benzene rings.

MATERIALS AND METHODS

Test compounds. The following compounds were purchased from Sigma (St. Louis, Mo.) or Aldrich (Milwaukee, Wis.) (the common names are followed by molecular weights of the compounds in parentheses): benzaldehyde (106); benzoic acid (122); acetylsalicylic acid (aspirin, 180); 2-hydroxybenzaldehyde (salicylaldehyde, 122); 2-hydroxybenzoic acid (salicylic acid, 138); 2-methoxybenzaldehyde (*o*-anisaldehyde, 136); 2-methoxybenzoic acid (*o*-anisic acid, 152); 3-hydroxybenzaldehyde (122); 3-hydroxybenzoic acid (138); 3-methoxybenzaldehyde (*m*-anisaldehyde, 136); 3-methoxybenzoic acid (*m*-anisic acid, 152); 4-hydroxybenzaldehyde (122); 4-hydroxybenzoic acid (138); 4-methoxybenzaldehyde (*p*-anisaldehyde, 136); 4-methoxybenzoic acid (*p*-anisic acid, 152); 2,3-dihydroxybenzaldehyde (138); 2,3-dihydroxybenzoic acid (154); 2,3-dimethoxybenzaldehyde (166); 2,3-dimethoxybenzoic acid (182); 2,4-dihydroxybenzaldehyde (β -resorcyaldehyde, 138); 2,4-dihydroxybenzoic acid (β -resorcylic acid, 154); 2,4-dimethoxybenzaldehyde (166); 2,4-dimethoxybenzoic acid (182); 2,5-dihydroxybenzaldehyde (gentisaldehyde, 138); 2,5-dihydroxybenzoic acid (gentisic acid, 154); 2,5-dimethoxybenzaldehyde (166); 2,5-dimethoxybenzoic acid (182); 2,6-dihydroxybenzoic acid (γ -resorcylic acid, 154); 2,6-dimethoxybenzaldehyde (166); 2,6-dimethoxybenzoic acid (182); 2-hydroxy-3-methoxybenzaldehyde (*o*-vanillin, 152); 2-hydroxy-3-methoxybenzoic acid (3-methoxysalicylic acid, 168); 2-hydroxy-

4-methoxybenzaldehyde (4-methoxysalicylaldehyde, 152); 2-hydroxy-4-methoxybenzoic acid (4-methoxysalicylic acid, 168); 2-hydroxy-5-methoxybenzaldehyde (5-methoxysalicylaldehyde, 152); 2-hydroxy-5-methoxybenzoic acid (5-methoxysalicylic acid, 168); 2-hydroxy-6-methoxybenzoic acid (6-methoxysalicylic acid, 168); 3,4-dihydroxybenzaldehyde (protocatechualdehyde, 138); 3,4-dihydroxybenzoic acid (protocatechuic acid, 154); 3,4-dimethoxybenzaldehyde (veratraldehyde, 166); 3,4-dimethoxybenzoic acid (veratric acid, 182); 3,5-dihydroxybenzaldehyde (138); 3,5-dihydroxybenzoic acid (α -resorcylic acid, 154); 3,5-dimethoxybenzaldehyde (168); 3,5-dimethoxybenzoic acid (182); 3-hydroxy-4-methoxybenzaldehyde (isovanillin, 152); 3-hydroxy-4-methoxybenzoic acid (isovanillic acid, 168); 4-hydroxy-3-methoxybenzaldehyde (vanillin, 152); 4-hydroxy-3-methoxybenzoic acid (vanillic acid, 168); 2,3,4-trihydroxybenzaldehyde (154); 2,3,4-trihydroxybenzoic acid (170); 2,3,4-trimethoxybenzaldehyde (196); 2,3,4-trimethoxybenzoic acid (212); 2,4,5-trimethoxybenzaldehyde (196); 2,4,5-trimethoxybenzoic acid (asaronic acid, 212); 2,4,6-trihydroxybenzaldehyde (phloroglucinocarboxaldehyde, 154); 2,4,6-trihydroxybenzoic acid monohydrate (188); 2,4,6-trimethoxybenzaldehyde (196); 3,4,5-trihydroxybenzaldehyde monohydrate (172); 3,4,5-trihydroxybenzoate methyl ester (gallic acid methyl ester, 184); 3,4,5-trihydroxybenzoic acid (gallic acid, 170); 3,4,5-trimethoxybenzaldehyde (196); 3,4,5-trimethoxybenzoic acid (212); 2-hydroxy-4,6-dimethoxybenzaldehyde (182); 3-hydroxy-4,5-dimethoxybenzoic acid (182); 3,4-dihydroxy-5-methoxybenzaldehyde (168); 4-hydroxy-2,6-dimethoxybenzaldehyde (182); 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde, 182); 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid, 198); and 5-hydroxy-3,4-dimethoxybenzaldehyde (182). Standard test compounds included *trans*-cinnamaldehyde, gentamycin, and chloramphenicol (Sigma). All compounds were purported to be 96 to 99% pure.

Test buffers. Phosphate-buffered saline (PBS, pH 7.0) was prepared by mixing dibasic sodium phosphate (100 mM) and monobasic sodium phosphate (100 mM) at a 2:1 ratio, diluting by half with H₂O, and adding NaCl (150 mM). For stronger buffering, the benzoic acids were prepared in 200 mM PBS (pH 7.0) buffer. For the pH study, the PBS buffer (pH 7.0) was adjusted to pH 8.4 with 5 N NaOH, and a 2 mM citric acid–150 mM NaCl buffer was adjusted to pH 3.7 or pH 5.4.

Test solutions. Test solutions or suspensions were prepared by adding 10 μ l of liquid test compound or 10 to 15 mg of solid test compound to 1 ml of buffer and vortexing for 1 min. If they were insoluble, the solutions were microwaved for 4 to 5 s on the high setting so that condensation on the tube was observed but volume was not lost. The samples were vortexed for 1 min and shaken.

Suspended stock solutions were shaken vigorously by hand in 1.9-ml sterile plastic snap-cap microcentrifuge tubes. A 200- μ l aliquot was added to 400 μ l of buffer and then shaken vigorously prior to the addition of the next aliquot. For *E. coli*, *S. enterica*, and *L. monocytogenes*, the samples were diluted threefold, and for *C. jejuni*, the samples were diluted fourfold for each of six dilutions, respectively. The antibiotic gentamycin was used as a positive control for *C. jejuni*, *E. coli*, and *S. enterica*, and the antibiotic chloramphenicol was used as a positive control for *L. monocytogenes*. On the basis of our extensive experience with cinnamaldehyde (14), this compound was also used as a positive control for *C. jejuni*, *E. coli*, and *S. enterica*.

Sources of bacteria. *C. jejuni* RM1221, *E. coli* O157:H7 strain RM1484, *L. monocytogenes* RM2199, and *S. enterica* ser-

ovar Hadar strain RM1309, obtained from contaminated food and clinical sources and described previously (14), were used in this study.

Bactericidal assays. The bactericidal assay was developed by modifying a previously described microtiter plate bactericidal assay (14). Briefly, *C. jejuni* was cultured on iron-supplemented brucella agar (BA) (Difco Laboratories, Sparks, Md.) in Hefty One-Zip Slider Freezer bags (Hefty, Lake Forest, Ill.) filled with a microaerophilic gas mixture (5% O₂, 10% CO₂, and 85% N₂) for 18 to 19 h at 42°C. The bags were filled with gas, the gas was expelled three times, and then the bags were sealed tightly to ensure the proper atmosphere. Streaked plates, rather than broth cultures, were used as the source of *C. jejuni*. *E. coli* and *S. enterica* were cultured in Luria-Bertani agar broth (Difco) for 16 to 18 h at 37°C, and *L. monocytogenes* was cultured in iron-supplemented brain heart infusion broth (Difco) for 16 to 18 h at 37°C.

A 1-ml sample of stationary-phase bacteria (*E. coli*, *S. enterica*, or *L. monocytogenes*) in broth was centrifuged, and the pellet was resuspended in 1 ml of sterile PBS. The sample's optical density at 620 nm (OD₆₂₀) was adjusted to ca. 0.8 with PBS. Each 10- μ l sample was added to 990 μ l of PBS (1:100), and then 80 μ l of the 1:100 sample was diluted in 20 ml of PBS to a final dilution of 1:25,000 containing ca. 1,500 to 2,000 cells.

Studies to optimize the bactericidal assay for the microaerophilic *C. jejuni* were carried out. Specifically, colonies on plates from 16- to 18-h cultures were sampled with a loop and suspended in 1 ml of PBS. The bacterial suspension was centrifuged, and the pellet was resuspended in PBS. The OD₆₂₀ of the suspension was adjusted to 1.0 to 1.3. The diluted sample (10 μ l) was added to 990 μ l of PBS (1:100), and then 20 μ l of the 1:100 sample was diluted in 20 ml of PBS to a final dilution of 1:100,000.

The assay mixture consisted of PBS buffer (pH 7.0), the test compound, and the bacteria. For the pH studies with *S. enterica*, 20 μ l of the 1:100 sample was diluted in 5 ml of PBS (pH 8.4 or pH 7.0) or citric acid-saline (pH 5.4 or pH 3.7).

Samples were prepared with sterile 96-well tissue culture microtiter plates (Nalge, Nunc International, Rochester, N.Y.). Each dilution was shaken prior to its addition to the wells. The addition of 100 μ l of PBS minus/plus test substance to the wells was followed by the addition of 50 μ l of bacterial suspension; thus, the original stock solution was diluted by one-third. Six wells were used for each control to obtain an average control value; one positive control (gentamycin, chloramphenicol, or *trans*-cinnamaldehyde) was represented in a dilution series across six wells, and 14 test compounds were represented in dilution series across the remaining wells. The wells on the plate were covered with a SealPlate cover (Marsh Bioproducts, Rochester, N.Y.), sealed on the outside edges with Parafilm, and then incubated with gentle shaking for 60 min at 37°C (42°C for *C. jejuni*). For the pH studies, *S. enterica* was incubated for 30 and 60 min at 37°C.

Following incubation, a 20- μ l sample from each well was spotted at the top of a square petri dish containing Luria-Bertani medium (for *E. coli* and *S. enterica*), brain heart infusion agar (for *L. monocytogenes*), or iron-supplemented BA (for *C. jejuni*). Six 20- μ l spots were spaced across the top—one for each dilution of test sample or six controls. The plates were placed uncovered in a biological safety hood until the sample liquid dried (ca. 10 min) and were then covered. *E. coli*, *S. enterica*, and *L. monocytogenes* plates were incubated overnight at 37°C. *C. jejuni* plates were placed in sealable bags filled with gas (5% O₂, 10% CO₂, and 85% N₂) and incubated at 42°C.

E. coli, *S. enterica*, and *L. monocytogenes* colonies were counted after 18 to 24 h. *C. jejuni* CFU in iron-supplemented BA

were usually small but were counted at 24 h. We also observed a purplish black staining of the iron-supplemented BA with some of the compounds. The benzaldehydes and gallic acid used in the pH studies were observed to be chromogenic at neutral and alkaline pHs. Experimental results were analyzed only when control counts were 60 to 200 CFU. In each case, at least two separate experiments, involving bacterial pellets processed from two different cultures, were performed.

Bactericidal activity. Bactericidal activity was defined as the percentage of test compound that kills 50% of the bacteria after 60 min (BA₅₀) and was determined as follows (14). Each compound was tested as a series of six dilutions from 0.00065 to 0.67% in the reaction mixture. The CFU counts for all experiments were transferred to a Microsoft Excel 8.0 Spreadsheet. The number of CFU from each dilution was matched with the average control value to determine the percentage of bacteria killed per well. Each of the dose-response profiles (percentage of test compound versus percentage of bactericidal activity) was examined graphically, and the BA₅₀ values were estimated by a linear regression (36). The lower the BA₅₀ value or the higher the 1/BA₅₀ value, the higher the activity. A BA₅₀ value of >0.67% considered to indicate inactivity.

RESULTS

Table 1 shows the BA₅₀ values for benzaldehyde, benzoic acid, and 68 derivatives substituted with OH, OCH₃, and both groups in various positions of the benzene ring. The observed solubilities and colors of these compounds are also given in the Table 1. Four types of preparations were observed: solutions; suspensions; milky, oily suspensions; and immiscible oils with rapid oil separation. The 5-s microwaving used to facilitate solubilization should not affect these compounds in view of reports that microwaves do not adversely affect the nutrition and safety of different categories of food ingredients (10).

Under the test conditions, benzoic acid was inactive against all four bacteria (BA₅₀ > 0.67). In contrast, benzaldehyde was highly active against *C. jejuni* (BA₅₀ = 0.045) and less active against the other three bacteria (with BA₅₀ values ranging from 0.36 to 0.48).

To facilitate discussion of the effects of structure on antimicrobial activity, Figure 2 shows plots of 1/BA₅₀ values (calculated from the BA₅₀ values listed in Table 1) for the 10 compounds that were most active against each of the four pathogens. Table 2 lists average BA₅₀ values for the 24 compounds active against all four species, and Table 3 presents data on the effects of pH on the bactericidal activities of selected compounds.

Figure 3 depicts so-called box-and-whisker plots, well-established statistical tools (26, 43) that allow the visualization of the trends and distributions of activities against the four pathogens (1/BA₅₀ values) for all 70 compounds listed in Table 1. The description of these results will be divided into three parts, covering monosubstituted, disubstituted, and trisubstituted compounds as listed in Table 1.

Monosubstituted benzaldehydes and benzoic acids.

2-Hydroxybenzaldehyde (salicylaldehyde) was quite active against all four bacteria, with BA₅₀ values ranging from 0.064 to 0.26. In contrast, 2-hydroxybenzoic (salicylic) acid

TABLE 1. Bactericidal activity of hydroxy- and methoxybenzaldehydes and benzoic acids against *C. jejuni*, *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica*

BA ₅₀ (mean ± SD) for bacterium ^a							
Chemical	Common name	<i>C. jejuni</i>	<i>E. coli</i>	<i>L. monocytogenes</i>	<i>S. enterica</i>	Solubility	Color
1. Benzaldehyde	Benzaldehyde	0.045 ± 0.03 ^b	0.48 ± 0.007	0.46 ± 0	0.36 ± 0.21	Suspension	Gray
2. Benzoic acid	Benzoic acid	>0.67 ^c	>0.67	>0.67	>0.67	Soluble	Colorless
Monosubstituted compounds							
3. Acetylsalicylic acid	Aspirin	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
4. 2-Hydroxybenzaldehyde	Salicylaldehyde	0.064 ± 0.03 ^d	0.14 ± 0.003	0.26 ± 0.10	0.20 ± 0.10	Suspension	Green
5. 2-Hydroxybenzoic acid	Salicylic acid	0.65 ± 0.13 ^d	>0.67	>0.67	0.61 ± 0.05	Soluble	Colorless
6. 2-Methoxybenzaldehyde	<i>o</i> -Anisaldehyde	0.23 ± 0.12	0.37 ± 0.21 ^e	0.66 ± 0.06	0.19 ± 0.03 ^e	Suspension	Gray
7. 2-Methoxybenzoic acid	<i>o</i> -Anisic acid	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
8. 3-Hydroxybenzaldehyde		0.20 ± 0.07 ^d	0.25 ± 0.13 ^d	0.37 ± 0.07	0.27 ± 0.014	Soluble	Green
9. 3-Hydroxybenzoic acid		>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
10. 3-Methoxybenzaldehyde	<i>m</i> -Anisaldehyde	0.091 ± 0.003	0.53 ± 0.08 ^e	>0.67	0.14 ± 0.01	Suspensions	Gray
11. 3-Methoxybenzoic acid	<i>m</i> -Anisic acid	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
12. 4-Hydroxybenzaldehyde		0.20 ± 0.063	0.41 ± 0.03	0.40 ± 0.05	0.31 ± 0	Soluble	Green
13. 4-Hydroxybenzoic acid		>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
14. 4-Methoxybenzaldehyde	<i>p</i> -Anisaldehyde	0.068 ± 0.05 ^e	0.37 ± 0.20 ^e	0.58 ± 0.06	0.30 ± 0.13 ^d	Suspension	Gray
15. 4-Methoxybenzoic acid	<i>p</i> -Anisic acid	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
Disubstituted compounds							
16. 2,3-Dihydroxybenzaldehyde		0.016 ± 0.006	0.093 ± 0.05 ^d	0.049 ± 0.04	0.11 ± 0	Soluble	Greenish yellow
17. 2,3-Dihydroxybenzoic acid		0.31 ± 0.06 ^d	>0.67	>0.67	>0.67	Soluble	Colorless
18. 2,3-Dimethoxybenzaldehyde		0.29 ± 0.007	0.54 ± 0.01 ^e	0.58 ± 0.04	0.31 ± 0.17 ^e	Insoluble	Colorless
19. 2,3-Dimethoxybenzoic acid		>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
20. 2,4-Dihydroxybenzaldehyde	β-Resoreylaldehyde	0.065 ± 0.06 ^b	0.14 ± 0.006 ^d	0.35 ± 0.01	0.098 ± 0	Soluble	Colorless
21. 2,4-Dihydroxybenzoic acid	β-Resoreylic acid	0.46 ± 0.09 ^d	>0.67	>0.67	>0.67	Soluble	Colorless
22. 2,4-Dimethoxybenzaldehyde		0.38 ± 0.03	>0.67	>0.67	>0.67	Insoluble	White
23. 2,4-Dimethoxybenzoic acid		>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
24. 2,5-Dihydroxybenzaldehyde	Gentisaldehyde	0.017 ± 0.0001	0.043 ± 0.001 ^d	0.063 ± 0.013	0.043 ± 0.001	Soluble	Greenish brown
25. 2,5-Dihydroxybenzoic acid	Gentisic acid	0.20 ± 0.20 ^d	>0.67	>0.67	0.48 ± 0.02	Soluble	Colorless
26. 2,5-Dimethoxybenzaldehyde		0.36 ± 0.007	>0.67	>0.67	0.40 ± 0.17	Insoluble	Yellow
27. 2,5-Dimethoxybenzoic acid		>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
28. 2,6-Dihydroxybenzoic acid		0.40 ± 0.08 ^d	>0.67	>0.67	0.47 ± 0.03	Soluble	Colorless
29. 2,6-Dimethoxybenzaldehyde	γ-Resoreylic acid	0.15 ± 0.08	0.66 ± 0.12 ^e	0.40 ± 0.21	0.56 ± 0.03	Insoluble	White
30. 2,6-Dimethoxybenzoic acid		>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
31. 3,4-Dihydroxybenzaldehyde	Protocatechualdehyde	0.0031 ± 0.0006	0.22 ± 0.15 ^d	0.33 ± 0.06	0.27 ± 0.007	Soluble	Brown
32. 3,4-Dihydroxybenzoic acid	Protocatechuic acid	0.11 ± 0.06 ^d	>0.67	>0.67	0.50 ^f	Soluble	Colorless
33. 3,4-Dimethoxybenzaldehyde	Veratraldehyde	0.48 ± 0.08	>0.67	0.29 ± 0.01	>0.67	Soluble	Colorless
34. 3,4-Dimethoxybenzoic acid	Veratric acid	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
35. 3,5-Dihydroxybenzaldehyde		0.19 ± 0.11 ^d	0.15 ± 0.03 ^h	0.065 ± 0.003	0.18 ± 0.11	Soluble	Red
36. 3,5-Dihydroxybenzoic acid	α-Resoreylic acid	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
37. 3,5-Dimethoxybenzaldehyde		0.088 ^f	>0.67	>0.67	0.65 ± 0.23	Suspension	Gray
38. 3,5-Dimethoxybenzoic acid		0.28 ± 0.24	>0.67	>0.67	>0.67	Soluble	Colorless

TABLE 1. Continued

BA ₅₀ (mean ± SD) for bacterium ^a							
Chemical	Common name	<i>C. jejuni</i>	<i>E. coli</i>	<i>L. monocytogenes</i>	<i>S. enterica</i>	Solubility	Color
39. 2-Hydroxy-3-methoxybenzaldehyde	<i>o</i> -Vanillin	0.012 ± 0.005	0.20 ± 0.13 ^e	0.081 ± 0.006	0.25 ± 0.14 ^d	Suspension	Yellow
40. 2-Hydroxy-3-methoxybenzoic acid	3-Methoxysalicylic acid	0.45 ± 0.07 ^d	>0.67	>0.67	>0.67	Soluble	Colorless
41. 2-Hydroxy-4-methoxybenzaldehyde	4-Methoxysalicylal.	0.0025 ± 0.002	0.43 ± 0.17 ^e	0.034 ± 0.002	0.23 ± 0.11 ^d	Insoluble	Brown
42. 2-Hydroxy-4-methoxybenzoic acid	4-Methoxysalicylic acid	0.53 ± 0.09 ^d	>0.67	>0.67	>0.67	Soluble	Colorless
43. 2-Hydroxy-5-methoxybenzaldehyde	5-Methoxysalicylal.	0.00066 ± 0.0008 ^d	0.070 ± 0.01 ^e	0.10 ± 0.02	0.19 ± 0.22 ^d	Suspension	Yellow
44. 2-Hydroxy-5-methoxybenzoic acid	5-Methoxysalicylic acid	0.057 ± 0.063	>0.67	>0.67	>0.67	Soluble	Brown
45. 2-Hydroxy-6-methoxybenzoic acid	6-Methoxysalicylic acid	0.18 ± 0.08 ^d	>0.67	>0.67	>0.67	Soluble	Colorless
46. 3-Hydroxy-4-methoxybenzaldehyde	Isovanillin	>0.67	>0.67	>0.67	0.61 ^f	Insoluble	White
47. 3-Hydroxy-4-methoxybenzoic acid	Isovanillic acid	>0.67	>0.67	>0.67	>0.67	Soluble	Brown
48. 4-Hydroxy-3-methoxybenzaldehyde	Vanillin	0.073 ± 0.02	0.62 ± 0.13 ^e	0.53 ± 0.02	0.63 ± 0.10 ^e	Soluble	Colorless
49. 4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
Trisubstituted compounds							
50. 2,3,4-Trihydroxybenzaldehyde		0.0037 ± 0.001 ^e	0.045 ± 0.005 ^d	0.20 ± 0.04	0.029 ± 0.002	Soluble	Brown
51. 2,3,4-Trihydroxybenzoic acid		0.046 ± 0.013 ^d	0.66 ± 0.10 ^e	>0.67	0.11 ± 0.02	Soluble	Colorless
52. 2,3,4-Trimethoxybenzaldehyde		0.53 ± 0.03	0.62 ± 0.28	0.64 ± 0.05	0.55 ± 0.06 ^e	Insoluble	White
53. 2,3,4-Trimethoxybenzoic acid		>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
54. 2,3,5-Trimethoxybenzaldehyde		>0.67	>0.67	>0.67	>0.67	Insoluble	White
55. 2,4,5-Trimethoxybenzoic acid	Asaronic acid	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
56. 2,4,6-Trihydroxybenzaldehyde		0.0080 ± 0.0076 ^e	0.055 ± 0.006 ^h	0.0094 ± 0.0004	0.031 ± 0.01	Insoluble	Red
57. 2,4,6-Trihydroxybenzoic acid		>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
58. 2,4,6-Trimethoxybenzaldehyde		0.011 ± 0.004	0.54 ^f	0.64 ± 0.06	>0.67	Insoluble	White
59. 3,4,5-Trihydroxybenzaldehyde		0.0026 ± 0.002 ^g	0.042 ± 0.01 ⁱ	>0.67	0.054 ± 0.04 ^j	Insoluble	Brownish green
60. 3,4,5-Trihydroxybenzoate methyl ester	Methylparaben	0.0093 ± 0.0001	0.37 ± 0.007	0.19 ± 0.20	0.30 ± 0.02	Soluble	Yellow
61. 3,4,5-Trihydroxybenzoic acid	Gallic acid	0.0049 ± 0.0003 ^d	>0.67	>0.67	0.038 ± 0.02 ^d	Soluble	Colorless
62. 3,4,5-Trimethoxybenzaldehyde		>0.67	>0.67	>0.67	>0.67	Insoluble	White
63. 3,4,5-Trimethoxybenzoic acid		0.43 ± 0	>0.67	>0.67	>0.67	Soluble	Colorless
64. 2-Hydroxy-4,6-dimethoxybenzaldehyde		0.038 ± 0.007	>0.67	>0.67	>0.67	Insoluble	White
65. 3,4-Dihydroxy-5-methoxybenzaldehyde		0.0033 ± 0.0013	0.29 ± 0.16 ^e	0.51 ± 0.05	0.30 ± 0.16 ^e	Soluble	Red
66. 3-Hydroxy-4,5-dimethoxybenzoic acid		0.047 ± 0.001	>0.67	>0.67	>0.67	Soluble	Colorless
67. 4-Hydroxy-2,6-dimethoxybenzaldehyde		0.0078 ± 0.001	0.046 ± 0.01 ^e	0.47 ± 0.11	0.052 ± 0.02 ^e	Suspension	Yellow
68. 4-Hydroxy-3,5-dimethoxybenzaldehyde	Syringaldehyde	0.031 ± 0.02	>0.67	>0.67	0.58 ± 0.21 ^e	Soluble	Yellow
69. 4-Hydroxy-3,5-dimethoxybenzoic acid	Syringic acid	>0.67	>0.67	>0.67	>0.67	Soluble	Colorless
70. 5-Hydroxy-3,4-dimethoxybenzaldehyde		0.37 ± 0.06	0.51 ± 0.12 ^e	0.38 ± 0.09	0.55 ± 0.12 ^e	Soluble	Yellow
Positive controls							
Cinnamaldehyde	Cinnamaldehyde	0.0027 ± 0.002 ^d	0.038 ± 0.01 ^d		0.02 ± 0.007 ^d	Suspension	Yellow
Gentamycin	Gentamycin	0.00015 ± 0.00003 ^d	0.00019 ± 0.0002 ^h		0.000042 ^h	Soluble	
Chloramphenicol	Chloramphenicol			0.015 ± 0.002 ⁱ		Soluble	

^a BA₅₀ values are for two separate experiments (*n* = 2) unless otherwise indicated. ^b *n* = 5. ^c *A* value of >0.67 indicates inactivity. ^d *n* = 4. ^e *n* = 4. ^f *n* = 1. ^g *n* = 11. ^h *n* = 6. ⁱ *n* = 10. ^j *n* = 8.

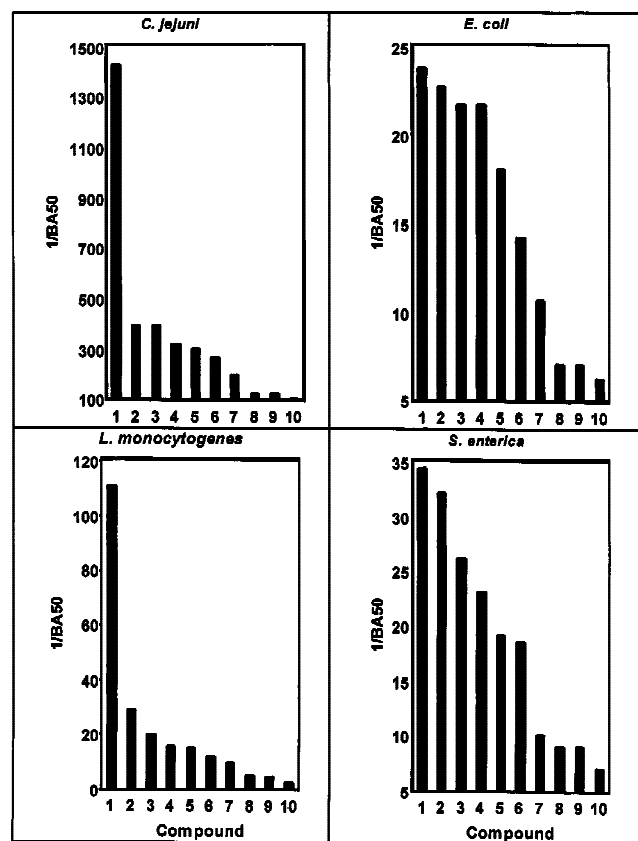


FIGURE 2. Comparison of bactericidal activities ($1/BA_{50}$ values) of the 10 compounds that were most active against each of pathogens. *C. jejuni*: (1) 2-hydroxy-5-methoxybenzaldehyde (highest level of activity); (2) 3,4,5-trihydroxybenzaldehyde; (3) 2-hydroxy-4-methoxybenzaldehyde; (4) 3,4-dihydroxybenzaldehyde; (5) 3,4-dihydroxy-5-methoxybenzaldehyde; (6) 2,3,4-trihydroxybenzaldehyde; (7) 3,4,5-trihydroxybenzoic acid; (8) 4-hydroxy-2,6-dimethoxybenzaldehyde; (9) 2,4,6-trihydroxybenzaldehyde; (10) 3,4,5-trihydroxybenzoate methyl ester. *E. coli*: (1) 3,4,5-trihydroxybenzaldehyde; (2) 2,3,4-trihydroxybenzaldehyde; (3) 4-hydroxy-2,6-dimethoxybenzaldehyde; (4) 2,5-dihydroxybenzaldehyde; (5) 2,4,6-trihydroxybenzaldehyde; (6) 2-hydroxy-5-methoxybenzaldehyde; (7) 2,3-dihydroxybenzaldehyde; (8) 2,4-dihydroxybenzaldehyde; (9) 2-hydroxybenzaldehyde; (10) 3,5-dihydroxybenzaldehyde. *L. monocytogenes*: (1) 2,4,6-trihydroxybenzaldehyde; (2) 2-hydroxy-4-methoxybenzaldehyde; (3) 2,3-dihydroxybenzaldehyde; (4) 2,5-dihydroxybenzaldehyde; (5) 3,5-dihydroxybenzaldehyde; (6) 2-hydroxy-3-methoxybenzaldehyde; (7) 2-hydroxy-5-methoxybenzaldehyde; (8) 3,4,5-trihydroxybenzoate methyl ester; (9) 2,3,4-trihydroxybenzaldehyde; (10) 2,4-dihydroxybenzaldehyde. *S. enterica*: (1) 2,3,4-trihydroxybenzaldehyde; (2) 2,4,6-trihydroxybenzaldehyde; (3) 3,4,5-trihydroxybenzoic acid; (4) 2,5-dihydroxybenzaldehyde; (5) 4-hydroxy-2,6-dimethoxybenzaldehyde; (6) 3,4,5-trihydroxybenzaldehyde; (7) 2,4-dihydroxybenzaldehyde; (8) 2,3,4-trihydroxybenzoic acid; (9) 2,3-dihydroxybenzaldehyde; (10) 3-methoxybenzaldehyde.

showed only borderline activity against the four bacteria. The corresponding 2-methoxybenzoic acid was inactive. Acetylation of the 2-OH group of benzoic acid forms the inactive acetylsalicylic acid (aspirin). Surprisingly, methylation of the 2-OH group of 2-hydroxybenzaldehyde to form 2-methoxybenzaldehyde (*o*-anisaldehyde) resulted in

TABLE 2. Bactericidal activities of compounds that were active against all four pathogens

Compound ^a	Average BA ₅₀ (%) ^b
2,4,6-Trihydroxybenzaldehyde (56)	0.026
2,5-Dihydroxybenzaldehyde (24)	0.042
2,3-Dihydroxybenzaldehyde (16)	0.067
2,3,4-Trihydroxybenzaldehyde (50)	0.069
2-Hydroxy-5-methoxybenzaldehyde (43)	0.090
2-Hydroxy-3-methoxybenzaldehyde (39)	0.136
4-Hydroxy-2,6-dimethoxybenzaldehyde (67)	0.144
2,5-Dihydroxybenzaldehyde (35)	0.146
2,4-Dihydroxybenzaldehyde (20)	0.163
2-Hydroxybenzaldehyde (4)	0.166
2-Hydroxy-4-methoxybenzaldehyde (41)	0.174
3,4,5-Trihydroxybenzoate methyl ester (60)	0.220
3-Hydroxybenzaldehyde (8)	0.272
3,4-Dihydroxy-5-methoxybenzaldehyde (65)	0.276
4-Hydroxybenzaldehyde (12)	0.330
4-Methoxybenzaldehyde (14)	0.330
Benzaldehyde (1)	0.336
2-Methoxybenzaldehyde (6)	0.362
2,3-Dimethoxybenzaldehyde (18)	0.430
2,6-Dimethoxybenzaldehyde (29)	0.442
5-Hydroxy-3,4-dimethoxybenzaldehyde (70)	0.452
4-Hydroxy-3-methoxybenzaldehyde (48)	0.463
2,3,4-Trimethoxybenzaldehyde (52)	0.585

^a Numbers in parentheses correspond to those listed in Table 1 for the same compounds.

^b The sum of the individual BA₅₀ values for the four pathogens listed in Table 1 divided by 4.

a decrease in, but not the elimination of, antibacterial activity (with BA₅₀ values ranging from 0.19 to 0.66).

With regard to the substitutions in the 3 positions of the benzene rings, we found that the BA₅₀ values for 3-hydroxybenzaldehyde ranged from 0.20 to 0.37, whereas 3-hydroxybenzoic acid was inactive. The BA₅₀ values for 3-methoxybenzaldehyde (*m*-anisaldehyde) ranged from 0.091 to >0.67, whereas 3-methoxybenzoic acid was inactive.

For compounds with substituents in the 4 position, the BA₅₀ values for 4-hydroxybenzaldehyde ranged from 0.20 to 0.41, and those for 4-methoxybenzaldehyde (*p*-anisaldehyde) ranged from 0.068 to 0.58. Both 4-hydroxy- and 4-methoxybenzoic acids were inactive.

Disubstituted benzaldehydes and benzoic acids. Disubstituted benzaldehydes and benzoic acids will be described in the following order: 2,3-, 2,4-, 2,6-, 3,4-, and 3,5-disubstituted derivatives with either OH or OCH₃ groups followed by compounds with both OH and OCH₃ substituents. Table 1 shows that 2,3-dihydroxybenzaldehyde was highly active against all four bacteria (with BA₅₀ values ranging from 0.016 to 0.11). The corresponding 2,3-dihydroxybenzoic acid was active only against *C. jejuni* (BA₅₀ = 0.31) and was inactive against the other three bacteria. Again, the activity level of 2,3-dimethoxybenzaldehyde (with BA₅₀ values ranging from 0.29 to 0.58) was

TABLE 3. Effects of pH and time on the bactericidal activities of gallic acid and selected hydroxy- and/or methoxy-substituted benzaldehydes against *S. enterica* for 30 and 60 min at 37°C

	BA ₅₀ (mean ± SD) for pH ^a							
	3.7 ^b				5.4 ^c			
	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min
3,4,5-Trihydroxybenzoic acid (gallic acid)	0.20 ± 0.07	0.19 ± 0.03	0.44 ± 0.06	0.26 ± 0.16	0.035 ± 0.01	0.0093 ± 0.0004		
4-Hydroxy-2,6-dimethoxybenzaldehyde	0.14 ± 0.15	0.024 ± 0.004	0.052 ± 0.005	0.018 ± 0.01	0.046 ± 0.01	0.029 ± 0.001		
2,3,4-Trihydroxybenzaldehyde	0.052 ± 0.01	0.037 ± 0.004	0.032 ± 0.001	0.022 ± 0.001	0.031 ± 0.001	0.029 ± 0.002		
2-Hydroxy-5-methoxybenzaldehyde	0.080 ± 0.05	0.036 ± 0.01	0.080 ± 0.03	0.060 ± 0.04	0.071 ± 0.03	0.028 ± 0.003		
2,5-Dihydroxybenzaldehyde (gentisaldehyde)	0.077 ± 0.03	0.034 ± 0.004	0.043 ± 0.002	0.033 ± 0.004	0.056 ^e	0.047 ^e		
2-Hydroxybenzaldehyde (salicylaldehyde)	0.10 ± 0.001	0.10 ± 0.01	0.42 ± 0.04	0.41 ± 0.01	0.12 ± 0.01	0.11 ± 0.01		

^a n = 2 unless otherwise noted.
^b 2 mM citric acid saline adjusted to pH 3.7.
^c 2 mM citric acid saline adjusted to pH 5.4.
^d 50 mM PBS adjusted to pH 7.0.
^e n = 1.

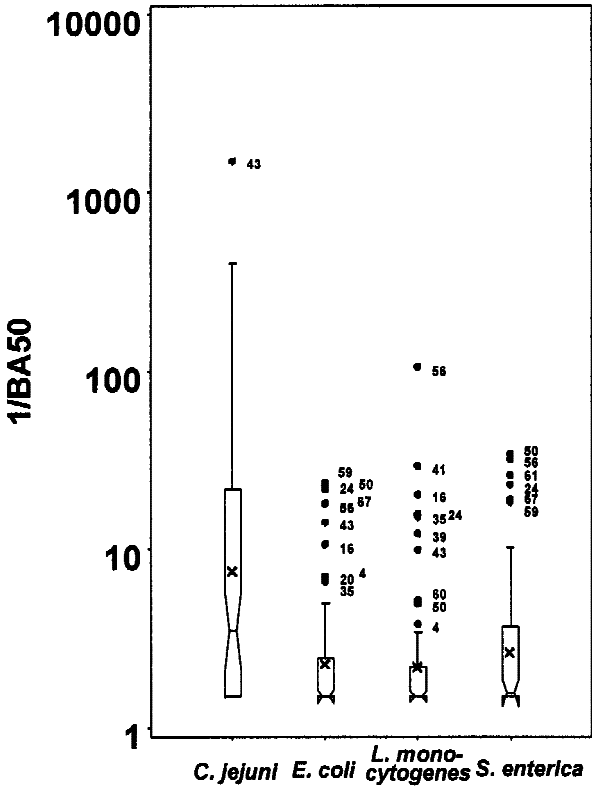


FIGURE 3. Box-in-whisker plots (26, 43) of the distribution of the activities of 70 compounds for each of the four pathogens. The box portion of the box-in-whisker plot delineates the median and the 25th and 75th percentiles of the distribution (bottom and top of the box, respectively). × represents the mean of the distribution. The whisker extends to the “upper fence,” which is 1.5 the interquartile range, i.e., 1.5 the distance from the median to the 75th percentile. Values above the upper fence are identified with numbers that correspond to those listed in Table 1.

lower than that for the corresponding dihydroxy compound. 2,3-Dimethoxybenzoic acid was inactive.

The data for 2,4-disubstituted compounds show that (i) the BA₅₀ values for 2,4-dihydroxybenzaldehyde ranged from 0.065 to 0.35; (ii) 2,4-dihydroxybenzoic acid was active against *C. jejuni* (BA₅₀ = 0.46) but not against the other three bacteria; and (iii) 2,4-dimethoxybenzaldehyde exhibited activity against *C. jejuni* (BA₅₀ = 0.38) but not against the other three bacteria. These results show that the activities of the 2,4-disubstituted benzaldehydes are similar to those of the corresponding 2,3 compounds. Both are more active than the monosubstituted compounds.

The activity level of 2,5-dihydroxybenzaldehyde (gentisaldehyde) (with BA₅₀ values ranging from 0.017 to 0.063) appears higher than that described above for the 2,3- and 2,4-disubstituted analogs. 2,5-Dihydroxybenzoic acid (gentisic acid) was active against *C. jejuni* (BA₅₀ = 0.20) and *S. enterica* (BA₅₀ = 0.48) but not against the other two bacteria. 2,5-Dimethoxybenzaldehyde was also active against *C. jejuni* (BA₅₀ = 0.36) and *S. enterica* (BA₅₀ = 0.40) but not against the other two bacteria. 2,5-Dimethoxybenzoic acid was inactive against all four bacteria.

The 2,6-dihydroxybenzaldehyde isomer was not available to us. Table 1 shows that the corresponding 2,6-dihy-

droxybenzoic acid (γ -resorcylic acid) was active against *C. jejuni* (BA_{50} = 0.40) and *S. enterica* (BA_{50} = 0.47) but not against the other two bacteria. 2,6-Dimethoxybenzaldehyde was active against all four bacteria (with BA_{50} values ranging from 0.15 to 0.66), whereas 2,6-dimethoxybenzoic acid was inactive against all four bacteria.

3,4-Dihydroxybenzaldehyde (protocatechualdehyde) was highly active against *C. jejuni* (BA_{50} = 0.0031) and less active against the other three bacteria (with BA_{50} values ranging from 0.22 to 0.33). 3,4-Dihydroxybenzoic acid (protocatechuic acid) was active against *C. jejuni* (BA_{50} = 0.11) and *S. enterica* (BA_{50} = 0.50) and inactive against the other two bacteria. 3,4-Dimethoxybenzaldehyde (veratraldehyde) was active against *L. monocytogenes* (BA_{50} = 0.29) and *C. jejuni* (BA_{50} = 0.48) and inactive against the other two bacteria. 3,4-Dimethoxybenzoic acid was inactive against all four bacteria.

3,5-Dihydroxybenzaldehyde showed high levels of activity against all four bacteria (with BA_{50} values ranging from 0.065 to 0.19). 3,5-Dimethoxybenzaldehyde was active against *C. jejuni* (BA_{50} = 0.088) and *S. enterica* (BA_{50} = 0.65) and inactive against the other two bacteria. 3,5-Dihydroxybenzoic acid was inactive against all four bacteria, whereas 3,5-dimethoxybenzoic acid was active against *C. jejuni* (BA_{50} = 0.28) and inactive against the other three species.

The data for the mixed hydroxy-methoxy substitutions show the following trends in activity. 2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin) showed substantial activity against all four bacteria (with BA_{50} values ranging from 0.012 to 0.25), whereas 2-hydroxy-3-methoxybenzoic acid was active only against *C. jejuni* (BA_{50} = 0.45). 2-Hydroxy-4-methoxybenzaldehyde was active against all four bacteria (with BA_{50} values ranging from 0.0025 to 0.43), whereas 2-hydroxy-4-methoxybenzoic acid was active only against *C. jejuni* (BA_{50} = 0.53). The BA_{50} values for the highly active 2-hydroxy-5-methoxybenzaldehyde ranged from 0.00066 for *C. jejuni* to 0.070 for *E. coli* to 0.10 for *L. monocytogenes* to 0.19 for *S. enterica*. 2-Hydroxy-5-methoxybenzoic acid was active against *C. jejuni* (BA_{50} = 0.057) and inactive against the other three bacteria, as was also the case for 2-hydroxy-6-methoxybenzoic acid (with a BA_{50} value of 0.18 for *C. jejuni*). 4-Hydroxy-3-methoxybenzaldehyde (vanillin) was active against *C. jejuni* (BA_{50} = 0.073) and less active against the other three bacteria (with BA_{50} values ranging from 0.53 to 0.63), whereas 3-hydroxy-4-methoxybenzaldehyde (isovanillin) was slightly active against *S. enterica* (BA_{50} = 0.61) and inactive against the other three pathogens. 4-Hydroxy-3-methoxybenzoic acid (vanillic acid) and 3-hydroxy-4-methoxybenzoic acid (isovanillic acid) were inactive against all four species.

Trisubstituted benzaldehydes and benzoic acids.

2,3,4-Trihydroxybenzaldehyde was highly active, as evidenced by BA_{50} values ranging from 0.0037 to 0.20 for the four bacteria. The corresponding 2,3,4-trihydroxybenzoic acid was active against *C. jejuni* (BA_{50} = 0.046) and *S. enterica* (BA_{50} = 0.11), slightly active against *E. coli*

(BA_{50} = 0.66), and inactive against *L. monocytogenes*. 2,3,4-Trimethoxybenzaldehyde exhibited a low level of activity (with BA_{50} values ranging from 0.53 to 0.64). 2,3,4-Trimethoxybenzoic acid was inactive against all four bacteria. Both 2,4,5-trimethoxybenzaldehyde and 2,4,5-trimethoxybenzoic acid were inactive against all four species.

2,4,6-Trihydroxybenzaldehyde was highly active against all four bacteria (with BA_{50} values ranging from 0.008 to 0.055), whereas 2,4,6-trimethoxybenzaldehyde showed a high level of activity against *C. jejuni* (BA_{50} = 0.011) and low levels of activity against the other three bacteria. The corresponding 2,4,6-trimethoxybenzoic acid was inactive against all four pathogens.

The data for the 3,4,5 series show that 3,4,5-trihydroxybenzaldehyde was inactive against *L. monocytogenes* but highly active against the other three organisms (with BA_{50} values ranging from 0.0026 to 0.054). Gallic acid (3,4,5-trihydroxybenzoic acid) was active against *C. jejuni* (BA_{50} = 0.0049) and *S. enterica* (BA_{50} = 0.038) but not against *E. coli* and *L. monocytogenes*. Esterification of the carboxyl group of gallic acid to form 3,4,5-trihydroxybenzoate methyl ester (methylparaben) resulted in BA_{50} values of 0.0093 for *C. jejuni*, 0.19 for *L. monocytogenes*, 0.30 for *S. enterica*, and 0.38 for *E. coli*. 3,4,5-Trimethoxybenzaldehyde was inactive against all four bacteria. 3,4,5-Trimethoxybenzoic acid was active against *C. jejuni* (BA_{50} = 0.43) and inactive against the other three species.

With respect to the mixed hydroxy-methoxy trisubstituted compounds, the results obtained in this study show that 2-hydroxy-4,6-dimethoxybenzaldehyde was active only against *C. jejuni* (BA_{50} = 0.038). 3,4-Dihydroxy-5-methoxybenzaldehyde was active against all four bacteria (with BA_{50} values ranging from 0.0033 to 0.51), and 3-hydroxy-4,5-dimethoxybenzoic acid was active only against *C. jejuni* (BA_{50} = 0.047). 4-Hydroxy-2,6-dimethoxybenzaldehyde was active against all four pathogens (with BA_{50} values ranging from 0.0078 to 0.47), 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) was active only against *C. jejuni* (BA_{50} = 0.031) and *S. enterica* (BA_{50} = 0.58), and 4-hydroxy-3,5-dimethoxybenzoic acid was inactive. 5-Hydroxy-3,4-dimethoxybenzaldehyde was active against all four species (with BA_{50} values ranging from 0.37 to 0.55).

To facilitate the application of the data to foods, Figure 2 shows the bactericidal activity levels of the 10 compounds that were most active against each of the four pathogens. For *C. jejuni*, BA_{50} values ranged from 0.0007 for 2-hydroxy-5-methoxybenzaldehyde to 0.0093 for 3,4,5-trihydroxybenzoate methyl ester; for *E. coli*, BA_{50} values ranged from 0.042 for 3,4,5-trihydroxybenzaldehyde to 0.160 for 3,5-dihydroxybenzaldehyde; for *L. monocytogenes*, BA_{50} values ranged from 0.009 for 2,4,6-trihydroxybenzaldehyde to 0.350 for 2,4-dihydroxybenzaldehyde; and for *S. enterica*, BA_{50} values ranged from 0.029 for 2,3,4-trihydroxybenzaldehyde to 0.140 for 3-methoxybenzaldehyde. To further facilitate the application of the data to foods contaminated with multiple strains of human pathogens, Table 2 lists the average BA_{50} values for the 24 compounds that were active against all four bacteria. These

BA₅₀ values range from 0.026 for 2,4,6-trihydroxybenzaldehyde to 0.585 for 2,3,4-trimethoxybenzaldehyde.

pH effects. Since pH can affect both the susceptibility of *S. enterica* to inactivation (22) and the ionization of the OH groups of the test compounds (15), additional exploratory studies were carried out to ascertain to what extent, if any, the pH of the medium would influence antibacterial activities against this pathogen. The limited data shown in Table 2 suggest that pH has a variable effect on the antimicrobial activities of the six compounds evaluated at pHs ranging from 3.7 to 7.0. Gallic acid was an exception. Its activity increased about 20-fold after 60 min when the pH was increased from 3.7 to 7.0. Attempts to determine activities at pH 8.4 were unsuccessful. The results were not reproducible, presumably because of the sensitivity of the microorganisms in the control samples to inactivation at the higher pH.

DISCUSSION

Structural and mechanistic aspects. The structure-function analysis of the compounds tested in this study contributes to efforts designed to elucidate the effects of structures and action mechanisms of antibacterial activities for this class of compounds. The requirement of the phenolic OH group for antimicrobial activity is strikingly demonstrated by the observation (44) that the phenolic OH group of carvacrol is essential for activity against *Bacillus cereus*, since cymene, a biological precursor of carvacrol lacking the OH group, had a much less extensive effect on the membrane potential associated with bactericidal activity than did carvacrol. In relevant studies, Ramos-Nino et al. (31) describe quantitative structure-activity relationships for several benzaldehydes with regard to the activities of these compounds against *L. monocytogenes*, *Salmonella* Enteritidis, and *Lactobacillus plantarum*. Multiregression analysis of the data and artificial neural network models showed that steric and polar parameters of the benzaldehydes were important but a lipophilic parameter was not. These findings suggest that rather than disrupting cell membranes, the compounds acted outside the cell. Additional chemical studies indicated that the benzaldehydes became covalently attached to surface SH groups of the bacterial cells (31).

Ramos-Nino et al. (30) also used mathematical relationships to describe the inhibition of a cocktail of 18 strains of *L. monocytogenes* in terms of steric and electronic effects associated with substituted benzoic and cinnamic acids and benzaldehydes. It is noteworthy that the mathematical model used to predict the activities of the pure compounds could not predict their activities in milk, meat, or soups. Activities against *Listeria* in these foods were generally lower than those observed in vitro. Payne et al. (28) also evaluated the antimicrobial activities of phenolic compounds against *L. monocytogenes* in milk.

Our results show that the aldehyde (CHO) group of benzaldehyde, but not the carboxyl (COOH) group of benzoic acid, is a structural feature that can contribute to antimicrobial activity in the absence of any phenolic OH groups on the benzene ring. Our data also show that the

monosubstituted phenolic derivatives of benzaldehyde are much more active than the parent unsubstituted compounds. The results of the present study also revealed the following order of bactericidal activity intensities elicited by the phenolic compounds (with some exceptions): trisubstituted OH > disubstituted OH > monosubstituted OH. For disubstituted derivatives, the 2-OH and 4-OH groups enhance the antimicrobial activities of benzaldehyde but not those of benzoic acid. Both the 2-OCH₃ and the 4-OCH₃ substitutions also increased activity levels for benzaldehyde. However, the increase for the methoxy groups was smaller than that for the corresponding hydroxy groups. Compounds with three methoxy groups were inactive. Compounds with mixed OH and OCH₃ groups exhibited variable results, i.e., in some cases the added methoxy groups enhanced activity and in others they had no effect.

The esterification of gallic acid to 3,4,5-trihydroxybenzoate methyl ester results in a significant increase in activity. This increase is presumably the basis for the extensive studies on the antimicrobial properties of the numerous synthetic gallic acid esters mentioned below. Antibacterial activities of gallic acid esters of different chain lengths are related to the length of the alkyl group of the ester side chain (16). The inhibition of oxygen use by methyl esters of hydroxycinnamates (alkacins) was found to be correlated with growth inhibition when hydroxycinnamates were tested against *Pseudomonas fluorescens* (2). These results suggest that bacteriostatic activities of phenolic compounds are based on cellular energy depletion and not on membrane-disrupting effects.

An examination of the trends in activities shown in Tables 1 and 2 and Figures 2 and 3 indicates similar distributions and clusterings of activities for *E. coli*, *S. enterica*, and *L. monocytogenes*. In contrast, levels of activity against *C. jejuni* were approximately 100 times as high as the observed levels of activity against the other three species. The plots also show a much broader distribution of activities against *C. jejuni*. We do not know the reasons for these differences. The summary of the activities for the 24 compounds that were active against all four species, presented in Table 2, will be helpful in devising antibacterial formulations with which to protect food and possibly also nonfood substrates against multiple pathogens.

Effect of pH on bactericidal activities of phenolic compounds. The ionization of a phenolic group to a phenoxide ion ($\text{ROH} \rightarrow \text{RO}^- + \text{H}^+$) is governed by its pK_a values as well as by the pH of the medium. The higher the pH, the more extensive the ionization. Since pK values of phenolic OH groups are usually >8 (15), the concentration of phenoxide ions is low at pH values of <7. Thus, if phenoxide ions rather than the protonated forms are the active antibacterial species, expectations are that activities of the phenolic compounds should increase with pH. It is therefore of inherent interest to find out whether the antibacterial activity levels of phenoxide ions (RO^-) are higher than those of the protonated forms (ROH). If they are, the enhancement of activity when the medium's pH is higher probably arises from the negative charge of the phenoxide

ions. These charges may alter the electrochemical balance in proximity to the microenvironment of the bacteria, facilitating cell death.

Exploratory studies summarized in Table 3 show that activity appears to increase as the pH increases from 3.7 to 7.0. However, except for gallic acid, this increase appears not to be significant in the narrow pH range studied. For gallic acid, the BA₅₀ value decreased from 0.20 at pH 3.7 to 0.035 at pH 7.0 after 30 min (a sixfold increase in activity). The corresponding change after 60 min was from 0.19 to 0.0093, a highly significant 20-fold increase in activity. Expectations were that this trend would accelerate at higher pH values. However, our efforts to measure antimicrobial activity at pH 8.4 were unsuccessful because the high pH killed significant amounts of the bacteria in the control sample. In related studies, we found that certain antibacterial phenolic compounds such as gallic acid are themselves unstable at high pHs (15). Since many foods, such as fruit and vegetable juices, vinegar salad dressings, and sweet and sour sauces, are on the acid side of the pH scale, the effect of pH on the antibacterial activities of phenolic compounds merits further study (9, 22, 32).

CONCLUSIONS

We have reported the antibacterial activities of a variety of phenolic benzaldehydes and benzoic acids against four human pathogens. The bactericidal assay was easy to perform, and the results were reproducible in assays with the same samples tested at different times. The ultimate goal of these studies is to develop a better understanding of the structural features of phenolic compounds that govern antibacterial activities as well as to devise food formulations that use the active compounds to reduce pathogens in foods, feeds, and possibly also in animals and humans after the consumption of contaminated foods. Studies involving foods need to take into account safety (38), solubility (Table 1), and sensory properties, including the color and flavor of the test compounds, as well as effects of the food matrix and of storage temperatures and times on antibacterial activities. The possible use of these compounds for the disinfection of nonfood substrates, e.g., meat cutting boards, also merits study.

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